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(71) Applicants *(for all designated States except US)*: THE UNIVERSITY OF NEW SOUTH WALES [AU/AU]; Anzac Parade, Kensington, NSW 2033 (AU). BURNS PHILP & COMPANY LIMITED [AU/AU]; 7 Bridge Street, Sydney, NSW 2000 (AU). BURNS PHILP RESEARCH & DEVELOPMENT PTY. LTD. [AU/AU]; 7 Bridge Street, Sydney, NSW 2000 (AU). COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). ARNOTT'S BISCUITS LIMITED [AU/AU]; 11 George Street, Homebush, NSW 2140 (AU). GIST-BROCADES AUSTRALIA PTY. LIMITED [AU/AU]; 9 Moorebank Avenue, Moorebank, NSW 2170 (AU). GOODMAN FIELDER INGREDIENTS LIMITED [AU/AU]; Level 4, 230 Victoria Road, Gladesville, NSW 2111 (AU). BROWN, Ian, Lewis [AU/AU]; 18 Valley Way, Gymea Bay, NSW 2227 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CONWAY, Patricia, Lynne [AU/AU]; 22 Goorawahl Avenue, La Perouse, NSW 2036 (AU). HENRIKSSON, Karl, Anders, Olof [SE/AU]; 10/174 Old South Head Road, Bellevue Hill, NSW 2023 (AU). McNAUGHT, Kenneth, John [AU/AU]; 10 Cottage Point Road, Cottage Point NSW 2084 (AU). WANG, Xin [CN/AU]; 4/121 Avoca Street, Randwick, NSW 2032 (AU).

(74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).

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(54) Title: SELECTION AND/OR ENHANCEMENT OF RESIDENT MICROORGANISMS IN THE GASTROINTESTINAL TRACT

(57) Abstract

Improved method of enhancing a population of one or more target microorganisms in the gastrointestinal tract of an animal, the improvement comprising providing to the animal a selected modified or unmodified resistant starch or mixtures thereof, such that the one or more microorganisms will selectively utilise the starch and/or increase in number and/or activity in the gastrointestinal tract, either uniformly throughout the gastrointestinal tract or at specific site or regions.

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Selection and/or Enhancement of Resident Microorganisms in the Gastrointestinal Tract

Technical Field

This invention relates to an improved method of enhancing a population of one or more target microorganisms in the gastrointestinal tract, especially the small intestine and the large bowel, of animals and humans.

Background Art

It is the contention of many scientists that the health and well being of people can be positively or negatively influenced by the microorganisms which inhabit the gastrointestinal tract, and in particular, the large bowel. These microorganisms through the production of toxins, metabolic by-products, short chain fatty acids, and the like affect the physiological condition of the host. The constitution and quantity of the gut microflora can be influenced by conditions or stress induced by disease, life style, travel, and other factors. If microorganisms which positively affect the health and well being of the individual can be encouraged to populate the large bowel, this should improve the physiological well being of the host.

The present inventors have realised that it would be desirable to provide a medium that would function to promote the growth and/or activity of target microorganisms in the gastrointestinal tract of animals including humans.

Disclosure of Invention

The present invention consists in an improved method of enhancing a population of one or more target microorganisms in the gastrointestinal tract of an animal, the improvement comprising providing to the animal a selected modified or unmodified resistant starch or mixtures thereof, such that the one or more microorganisms will selectively utilise the starch and/or increase in number and/or activity in the gastrointestinal tract.

The target population of microorganism may be enhanced throughout the gastrointestinal tract of the animal or targeted at specific sites of the gastrointestinal tract. It will be appreciated that the present invention will be suitable for any animal that requires alteration of its gastrointestinal flora. The present method is particularly suitable for use in humans.

The starches suitable include resistant or high amylose starches and modified forms thereof. The animal or human may be fed the selected resistant starch or the starch may be incorporated in a probiotic composition.

As used in this specification, "resistant starch" includes those forms defined as RS1, RS2, RS3 and RS4 as defined in Brown, McNaught and Moloney (1995) Food Australia 47: 272-275. Either modified or unmodified resistant starches or mixtures thereof are used in this invention. The advantage of resistant starch is that it is largely not degraded until it reaches the large bowel. Therefore it provides a readily available substrate for fermentation by the target microorganisms as soon as they arrive in the large bowel. In both cases, a preferred form of resistant starch is a high amylose starch particularly high amylose starches as disclosed and taught in WO 94/03049 and WO 94/14342, the contents of which are incorporated into this specification for the purposes of convenient cross-reference.

In WO 94/03049 and WO 94/14342, high amylose starches are disclosed which are resistant starches and include maize starch having an amylose content of 50% w/w or more, particularly 80% w/w or more, rice or wheat starch having an amylose content of 27% w/w or more and; particular granular size ranges of starches having an amylose content of 50% or more and enhanced resistant starch content, these starches including maize, barley, and legumes. This invention is not, however, limited to these forms of resistant starch. For example, other forms of resistant starch are derived from sources such as bananas and tubers such as potatoes and modified forms thereof.

It may be advantageous to also chemically modify the starch to, for instance, alter the charge density or hydrophobicity of the granule and/or granule surface to enhance the attachment compatibility between the microorganism and the resistant starch. Chemical modifications, such as etherification, esterification, acidification and the like are well known in this art as being suitable chemical treatments.

To modify the degree of enzyme susceptibility of the resistant starch the conformation or structure of the starch can be altered. Examples include acid or enzyme thinning and cross bonding using difunctional reagents.

The starches may be modified physically by, for example, crystallisation.

It is also within the scope of this invention to subject enzymatically treated resistant starches to chemical modification as described above.

As used herein, Hi-maize™ (trade mark) refers to a high amylose starch obtained from Starch Australasia Limited.

In order that the present invention may be more clearly understood, preferred forms thereof will be described with reference to the following figure and examples.

Brief Description of Drawings

Figure 1 shows comparison of the co-culturing of *Lactobacillus acidophilus* with *Bifidobacterium* strain X8AT2 in glucose and amylose starch medium.

Figure 2 shows enumeration of number of bifidobacteria in starch based medium inoculated with human faecal homogenates and incubated anaerobically at 37°C for 12 hours. Individual starches according to the description in Table 4.

Figure 3 shows enumeration of number of amylolytic bacteria in starch based media inoculated with human faecal homogenates and incubated anaerobically at 37°C for 12 hours. Individual starches as in Table 4.

Figure 4 shows enumeration of major bacterial groups in stomach contents from mice on various starch based diets (Table 4).

Figure 5 shows enumeration of major bacterial groups in ileal contents from mice on various starch based diets (Table 4).

Figure 6 shows enumeration of major bacterial groups caecal contents from mice on various starch based diets (Table 4)

Figure 7 shows enumeration of major bacterial group in colon contents from mice on various starch based diets (Table 4).

Figure 8 shows the total anaerobic microbial population of ileal origin, 9 hours post inoculation in media containing starch nos 4, 6, 8, 9 and glucose.

Figure 9 shows the total anaerobic microbial population of caecal origin, 9 hours post inoculation in media containing starch nos 4, 6, 8, 9 and glucose.

Modes for Carrying Out the Invention

Example 1

By measuring the amylase activity of specific intestinal bacteria when grown in standard laboratory medium containing glucose, starch (amylopectin) or resistant starch (amylose) added to a defined medium (composition included in Table 1 at a final concentration of 10 mg/ml), one can show that many of the intestinal bacteria produce amylase which can utilise the resistant starch (Table 2). In addition, the specific growth rates when six different intestinal bacteria were grown on glucose, amylose, amylopectin, Hi-maize™ and carboxymethylated resistant starch were determined (Table 3). The various bacteria tested grew at very different rates to each other, indicative that individual bacterial groups or species will be selectively enhanced by the form of starch used.

Table 1. Composition of medium used for growing intestinal strains of bacteria.

Ingredient	Amount
Bacteriological peptone	7.5g
Yeast extract	2.5g
Tryptone	5.0g
Starch	10.0g
K_2HPO_4	2.0g
KH_2PO_4	1.0g
$NaHCO_3$	0.2g
$NaCl_2$	2.0g
$MgCl_2$	0.2
$CaCl_2$	0.2g
$MnCl_2$	0.02g
$CoCl_2$	0.02g
Cystein	0.5g
$FeSO_4$	0.005g
Tween 80	2 ml
Hemin	0.005g
Vit B_{12}	0.001g
Vit K	0.0005g
Water (final volume)	1 litre

Table 2. Amylase activity after growth of intestinal isolates on starch and resistant starch.

Bacteria	Glucose	Amylopectin	Amylose
1. Supernatant			
Cl. butyricum	0.592	0.987	0.317
Bact. fragilis	0.064	0.563	0.927
Bif. bifidum	0.506	0.131	0.293
Bif. pseudolongum	0.087	0.542	0.423
E. limosum	0.202	0.568	0.794
Bact. vulgatus	0.196	0.602	0.380
2. Cell Extract			
Cl. butyricum	0.000	0.000	0.021
Bact. fragilis	0.045	1.038	2.018
Bif. bifidum	0.295	4.271	9.270
Bif. pseudolongum	0.664	3.855	12.685
E. limosum	0.375	0.491	0.039
Bact. vulgatus	0.229	1.644	3.381

Table 3. Specific growth rates.

Bacteria	Glucose	Amylose	Amylopectin	Hi Maize	Modified starch A 955 D2
Cl. butyricum	1.348	1.091	1.326	1.071	0.986
Bif. bifidum	0.8.6	0.509	0.721	0.746	0.704
Bif. pseudolongum	0.807	0.575	0.712	0.692	0.658
Bact. vulgatus	0.834	0.331	0.680	0.501	0.598
Bact. fragilis	0.645	0.355	0.490	0.398	0.448
E. limosum	0.570	0.338	0.632	0.421	0.320

Example 2

A number of modifications of the resistant starch (Hi-maize™) (Table 4) were used in the defined growth medium presented in Table 1. The intestinal isolates were then inoculated and the starch concentration determined after 22 h incubation as an indication of the extent of utilisation. Total carbohydrate was estimated using phenol-sulphuric acid assay. Surprisingly, a modification often resulted in altered utilisation of the starch as can be seen in Table 5.

Table 4. Starch identification

Starch	Destination	Identification	Analysis
1	A939 (D19)	Hydroxypropylated	DS* = 0.13
2	A938 (C79)	Acetylated	Acetyl value = 2.69%
3	A961 (D8)	Octenyl succinated	OSA value = 4.73%
4	A955 (D2)	Carboxymethylated	Carboxyl value = 1.0%
5	A960 (D7)	Succinated	Succinyl value = 3.97%
6	HA 008 (D2118)	Unmodified	-
7	A993 D42	Succinated	Succinyl value = 4.1%
8	A956 (D1)	Carboxymethylated	Carboxyl value = 2.0%
9	A995 (D57)	Acetylated	Acetyl value = 4.0%
10	A965 (D9)	Hydroxypropylated	DS = 0.13

* degree of substitution

Table 5. Concentration of starch after incubation for 22 hours.

	Starches									
Bacteria	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Cl. butyricum	3.364	1.829	2.354	3.714	1.418	2.175	2.980	3.121	2.648	-
Bif. pseudolongum	5.532	4.029	5.091	3.658	6.843	5.308	5.130	4.157	4.899	4.463
Bif. bifidum	5.245	4.132	7.035	4.503	5.437	4.950	4.375	4.720	5.091	5.454
Bact. fragilis	4.081	5.372	7.995	4.669	7.547	6.971	6.140	5.001	7.547	5.660
Bact. vulgatus	-	8.570	7.419	6.843	8.954	9.210	10.489	6.108	6.332	6.908
Bif. strain X8AT2	10.106	6.492	10.00	5.532	6.268	7.931	9.850	6.843	5.820	5.916
Lact. acidophilus	8.75	10.501	10.50	10.50	92.84	10.50	10.07	10.50	10.50	95.54
Lact. helviticus	52.76	10.50	10.50	10.50	10.50	10.50	10.50	99.68	10.50	10.50

Starch concentrations after 22 hours incubation (mg/ml)

1: A. 939 (D19) Hydroxypropylated; 2: A. 938 (C79) Acetylated; 3: A.961 (D8) Octenyl succinated; 4: A.955 (D2) Carboxymethylated; 5: A.960 (D7) Succinated; 6: HA 008 (D2118) Unmodified; 7: A993 D42 Succinated; 8: A956 (D1) Carboxymethylated; 9: A995 (D57) Acetylated; 10: A965 (D9) Hydroxypropylated;

Example 3

The effect of co-culture with amylolytic *Bif.* X8AT2 on the growth of *Lactobacillus sp* in the amylose starch medium

The growth of *Lact. acidophilus* in the Hi-maize™ containing medium with or without the present of Bif. X8AT2 was compared. The growth medium contained 1% Hi-maize™ starch or glucose as the growth carbon and energy source. The medium was autoclaved at 121°C for 15 minutes and strictly anaerobic conditions were used during the medium preparation. Overnight cultures (0.1 ml) of *Lact. acidophilus* and Bif. X8AT2 were inoculated into the serum tubes containing either glucose or Hi-maize™ starch based media. For the control *Lact. acidophilus* only was inoculated into the serum tubes. The tubes were then incubated at 37°C for 24 hours. Samples were taken at 0, 2, 4, 6, 8, 10, 12 and 24 hours to enumerate the population of *Lact. acidophilus* by using standard series dilution method. The population of *Lact. acidophilus* was expressed as CFU/ml on MRS agar plates.

Since *Lact. acidophilus* can not degrade Hi-maize™ starch, the growth of *Lact. acidophilus* in the defined medium containing Hi-maize™ starch as the sole carbon source was very slow and resulted in low biomass. The improvement of the growth of *Lact. acidophilus* in Hi-maize™ medium was observed when the strain was co cultured with the Hi-maize™ starch-utiliser, Bif. strain X8AT2 (Fig. 1). As can be seen in Figure 1, a synergistic effect is demonstrated when the Bifidobacterial strain is inoculated with the *Lactobacillus*.

Example 4

Mice were fed either normal mouse diet or a prepared diet containing either waxy starch, Hi-maize™ or modified Hi-maize™ (carboxymethylated) and were orally dosed with 200 microlitres of *Bifidobacterium sp* strain X8AT2 or *Bifidobacterium bifidum* cultures. The composition of the mouse prepared diet is included in Table 6. Faecal samples were collected after continuous feeding from day 3 to day 8 of the diet plus the bifidobacteria. The major bacterial groups were enumerated using selective media and the total bacteria output for the groups were calculated. As can be seen in Table 7, *Bacteroides* numbers were enhanced significantly in mice when they were fed a modified resistant starch plus bifidobacteria compared to controls, which include mice fed resistant starch

plus bifidobacteria. While it is established that *Bacteroides* of intestinal origin can ferment both starch (amylopectin) and resistant starch (amylose) reviewed by Salyers and Leedle (Salyers & Leedle, 1983), it is surprising to discover that a carboxymethylated amylose can significantly increase growth of the *Bacteroides*.

Table 6. Diets for mice probiotic feeding experiments.

Test Groups	A	B	C	D	E
Starch	Waxy	HA	Carboxy -methyl	HA	None
	400	400	400	400	
Casein	200	200	200	200	
Canola oil	25	25	25	25	
Sunflower oil	25	25	25	25	
Sucrose	150	150	150	150	
Wheat bran	100	100	100	100	
Gelatin	20	20	20	20	
Mineral mix	67	67	67	67	
Vitamin mix	13	13	13	13	
Methionine	2	2	2	2	
Bacterial strain	X8AT2	X8AT2	X8AT2	None	X8AT2

Waxy=waxy maize; HA=High amylose starch; Carboxy-methyl=Carboxymethylated high amylose starch. All weights are in grams. Bacterial cultures (100 microlitres per day) were orally ingested by the mice with starch containing meals.

Table 7. *Bacteroides* population in mice feeding study (total bacteria output/per day per mice).

	Starches				
	Group A	**Group B**	**Group C**	**Group D**	**Group E**
Bacteroides	9.163 ± 0.42	8.961 ± 0.40	9.952 ± 0.357	8.961 ± 0.576	8.463 ± 0.569
Mean Diff. (compare to group A)		A-B: 0.202	A-C: -0.789	A-D: 0.202	A-E: 0.699
F-test		none	p<0.05	none	p<0.05
Mean Diff. (compare to group E)	A-E: -0.699	E-B: -0.498	E-C: -1.489	E-D: -0.497	
F-test	p<0.05	p<0.05	p<0.05	p<0.05	

Group A: Waxy starch plus X8AT2 -- *Bifidobacteria* human isolates;
Group B: Hi-maize™ starch plus X8AT2;
Group C: Carboxymethylated resistant starch plus X8AT2;
Group D: Hi-maize™ starch plus *Bif. bifidum*;
Group E: Normal mice diet plus X8AT2

Example 5

a) Four groups of six mice (Balb/c, SPF) were continuously fed with semisynthetic diets for 4 weeks. Group A received 40% waxy starch in the diet, and groups C and E had 40% modified starches D2 and D57, respectively in their diets. Group D was the Hi-maize™ starch group and group B was assigned as the control to be fed with normal mice diet. Two faecal samples were collected at the end of experimental period (4 weeks) to enumerate the population of Bifidobacterium by using propionic acid agar. Bifidobacteria were further identified by cell morphology under light microscopy. The population of Bifidobacterium was expressed as total output per day per mice.

The results from three experiments indicated that the specific pathogen free (SPF) mice used in the experiment were free of detectable bifidobacteria ($<10^3$) and continued to be so for the 2 months as control animals (Table 8). It is very surprising, however, to find that when the mice shifted from normal mice diet to the starch diets, the population of bifidobacteria increased significantly. The degree of increase depended on the type of starch incorporated into the diets. Hi-maize™ starch diet yielded the greatest numbers of native bifidobacteria in the mice faeces, followed by the waxy starch diet. Modified Hi-maize™ starch D57 demonstrated better results in the stimulation of the growth of bifidobacteria than modified Hi-maize™ starch D2. The results from previous experiments indicated that D2 starch mainly sustained the good growth of Bacteroides. The statistical analysis of the data is also presented in Table 8.

After the first stage of experiment in which the mice were fed with the experimental diet for 4 weeks, 200 ul of Bif. X8AT2 was orally dosed into mice for 5 days. Numbers of *Lactobacillus* from all of groups were quantified in the mice faeces at both stages of experiments by using Rogosa agar. The cell morphology of *Lactobacillus* were also checked under phase-contract microscopy.

It can be seen that the highest fermentability of starch was detected with *Cl. butyrium*. *Bif. bifidum* and *Bif. psuedologum* are also capable of hydrolysing all of the starches, while human isolate Bif. X8AT2 preferred starch nos. 2, 4, 5, 8, 9 and 10. *Bact. fragilis* has a stronger amylolytic capability to degrade starches than *Bact. vulgatus*. The poorest genus is

Lactobacillus, since both strains tested could only partially utilise the modified Hi-maize™ starch 1.

All of the mice were heavily colonised with dense populations of Lactobacillus. The influence of diets on faecal population of Lactobacillus is shown in Table 9. In general, none of the starch diets supported the increased growth of native *Lactobacillus*, in comparison with normal mice diets. Particularly low numbers of Lactobacillus were detected in the groups of mice fed with modified starches D2 and D57. The population of *Lactobacillus*, however, increased in the group of mice fed with Hi-maize™ diet when amylolytic bifidobacterial strain X8AT2 was associated with the mice.

Table 8. Native population of Bifidobacteria in mice fed with different starches diets (CFU log 10/g faeces)

	Starches				
	Group A	Group B	Group C	Group D	Group E
Bifidobacterium	7.48 ± 0.481	0 ± 0	1.475 ± 2.174	8.235 ± 0.46	6.432 ± 0.566
Positive mice in the test group	6/6	0/6	2/6	6/6	6/6
Mean Diff. (compare to group A) F-test		A-B: 7.47 $p<0.05$	A-C: -6.005 $p<0.05$	A-D: -0.755 none	A-E: -1.048 none
Mean Diff. (compare to group B) F-test	B-A: -7.48 $p<0.05$		B-C: - 1.475 $p<0.05$	B-D:-8.235 $p<0.05$	B-E: -6.432 $p<0.05$

Group A: Waxy starch
Group B: Normal mice diet
Group C: Carboxymethylated amylose starch
Group D: Hi-maize™ starch
Group E: Acetylated maize starch

Table 9. Lactobacillus population in the mice fed with different starches diets (CFU log 10/g wet faeces)

	Starches				
	Group A	**Group B**	**Group C**	**Group D**	**Group E**
Lactobacillus					
Period 1: Fed with experimental diets for 4 weeks	7.596 ± 0.477	8.113 ± 0.532	7.423 ± 0.295	7.858 ± 0.367	7.309 ± 0.326
Mean Diff. (compare to group B)	B-A: 0.517		B-C: -0.690	B-D: -0.255	B-E: -0.804
F-test	none		$p<0.05$	none	$p<0.05$
Period 2: Experimental diets plus Bifidobacterium X8AT2	7.823 ± 0.397	7.782 ± 0.477	7.501 ± 0.319	8.031 ± 0.529	7.451 ± 0.673
Mean Diff. (compare to group D)	D-A: 0.208	D-B: 0.249	D-C: 0.531		D-E: -0.580
F-test	none	none	$p<0.05$		$p<0.05$

Example 6

a) Material from human colon was diluted Wilkins Chargren broth (1:1000).

The mixtures were incubated 37^{0}C for 24 h and sampled at 0, 3, 6, 9, 12 and 24 h post inoculation.

The type of resident starch or modifications thereof will induce an alteration or stimulation of resident microbes. After 9 h incubation, Starch nos. 8 and 9, induced an increase in the bifidobacterial population (Figure 2) followed by the bifidobacterial populations of cultures supplemented with Starch no. 1, 2, 10 and 7. Cultures supplemented with Starch no. 6 were less benefited, resulting in a relatively poor development of the bifidobacterial population. Starch no. 3 had only a moderate beneficial effect on bifidobacterial growth.

A large stimulation of the amylolytic microbial population (Figure 3) was detected when either Starch nos. 8, 4, 10 or 9 were used as a source of carbon. In contrast, poor development of the bifidobacterial population was noted in cultures supplied with Starch nos. 6, 3, 7 and 5. A close correlation between growth response of amylolytic and bifidobacterial populations was noted (Figures 2 and 3).

Example 7

Degradation of Starch nos. 1-10 by human faecal microorganisms

The degradation of resistant starch and modifications thereof (Table 4) by human faecal microbes was studied. After 12 and 24 h incubation of faecal homogenates in media based on the starches in Table 4 the various degree of utilisation was determined (Table 10). There was a great variation in resistance to degradation. Starch nos. 1 and 8 were most efficiently degraded by the human faecal microbiota, which resulted in 0.31 and 1.8%, respectively starch remaining in the cultures 24 h post inoculation. Starch nos. 7 and 9 were less efficiently degraded, giving about 9% remaining starch in the final culture 24 h post inoculation. The most resistant starch was Starch no. 6. The difference in resistance to degradation was even more significant in cultures incubated for 12 h. At this point (12 h), six starches were assayed: Starch nos. 1, 4, 6, 7, 8 and 9. Starch no. 1 was of the starches the most easily degraded (2.74% remaining), followed by Starch no. 8 (5.3%). Starch no. 4 (23.4%), Starch no. 9 (44.5%), Starch no. 7 (79.2%) and Starch

no. 6, the one most resistant to degradation. No degradation of starch no. 6 could be detected 12 h post inoculation (Table 10).

Table 10. Degradation of Starch nos. 1-10 by human faecal microorganisms.

Type of Starch (Table 4)	Residual starch (%)	
	12 h post inoculation	24 h post inoculation
1	2.73±0.46	0.31±0.10
2	N/A	7.07±1.24
3	N/A	8.57±1.08
4	23.4±4.72	5.59±1.73
5	N/A	11.8±2.86
6	119.±17.4	29.9±8.57
7	79.2±11.3	9.01±2.85
8	5.26±1.48	1.76±0.34
9	44.5±1.58	7.55±0.95
10	N/A	9.38±1.80

Example 8

Hi-maize™ can be modified to various levels with chemical reagents, such as acetic anhydride. The degree of susceptibility to in vitro digestion by bacterial alpha-amylase and amyloglucosidase of Hi-maize™ and three acetylated starches from Hi-Maize™ was ascertained using the Megazyme Total Starch Assay Procedure (AA/AMG 6/95). Each starch was solubilised and the enzyme resistant "residue" recovered by centrifugation. The residue was then solubilised using DMSO and assayed as per the Megazyme resistant starch method. The results are shown in Table 11.

Table 11. Resistance of acetylated Hi-maize™ starch to amylase digestion

Starch type	Amylose content (%) dsb*	Acetyl value (%) dsb	Enzyme solubilised starch (%) dsb	Starch residue (%) dsb
Hi-maize™	85	0	93.8	6.2
Starch A	-	2.85	66.5	33.5
Starch B	-	4.39	58.5	41.5
Starch C	-	7.72	35.5	64.5

* dry solids basis

Table 12. Degradation of Starch nos. 1-10 by human faecal microorganisms [Percentage starch degraders of total faecal population growing on amylose plates (Sigma)] at various times post inoculation.

	Amylolytic isotates in percentage of total CFU			
	3 h	6 h	9 h	12 h
Starch 1	100	56	56	30
Starch 2	**90**	**36**	**69**	**65**
Starch 3	80	35	28	12
Starch 4	**87**	**35**	**61**	**29**
Starch 5	81	50	54	28
Starch 6	88	58	16	7
Starch 7	63	47	48	10
Starch 8	72	66	67	56
Starch 9	**77**	**75**	**80**	**65**
Starch 10	72	73	58	21

Example 9

This example demonstrates that various modifications of resistant starch as presented in Table 4 induce the development of microbes with varying amylolytic activity. (Starch no. 1 and 8 are soluble and could not be assessed in this study). This was assessed by relating the number of isolates that produced clearing zones on amylose agar to the total population (CFU) on amylose plates (in % of total), and the degree of amylolytic activity expressed by amylolytic isolates. This was assessed by measuring clearing zones developed around colonies with amylolytic activity. There was a great variation in capacity the human faecal microbiota to degrade the different starches. Starch nos. 2 and 3 were degraded by the highest percentage of the population (65%) followed by Starch no 8 which was degraded by 56% of the population (Table 12). Starches 3 and 6 were degraded by only 12% and 7% respectively.

Production of Short Chain Fatty Acids (SCFA)

Compared to the glucose control, the addition of starches (except Starch no. 11) resulted in a significant increase in the production of all investigated SCFA's.

The production of n-butyric acid was greatest in media containing Starch no. 8, followed by Starch no. 4, Starch no. 5, Starch no. 2, Starch no. 6 and media containing Starch no. 10 (Table 13).

The production of acetic acid was greatest in media containing Starch no. 8, followed by Starch no. 1, Starch no. 2, Starch no. 10, Starch no. 5 and media containing Starch no. 9.

The production of propionic acid was greatest in media containing Starch no. 8, followed by Starch no. 3, Starch no. 9, Starch no. 6, Starch no. 4 and media containing Starch no. 2.

The production of iso-butyric acid was greatest in media containing glucose, followed by Starch no. 7 and Starch no. 3. Iso-butyric acid could not be detected in cultures supplied with any other starches.

The production of iso-valeric acid was greatest in media containing Starch no. 6, followed by media containing Starch no. 4, Starch no. 9, Starch no. 8, Starch no. 5 and glucose.

Starch no. 8 promoted production of all major SCFA's (acetic, propionic and butyric acid), more that any of the other starch, that resulted

in a butyric acid concentration that was about 1.5 times greater than for Starch no. 3 (Table 13).

Table 13. Production of Short Chain Fatty Acids from Starch nos. 1-11 and glucose, 24 h post inoculation with human faecal material.

	Short Chain Fatty Acid (mM)				
Type of carbon source	Acetic	Propionic	iso-Butyric	n-Butyric	iso-Valeric
Starch 1	40.7±4.17	15.4±1.21	0	11.5±2.44	0.18±0.37
Starch 2	37.9±0.44	15.8±0.11	0	9.66±0.37	0
Starch 3	35.4±0.95	18.5±0.62	0.48±0.95	8.28±0.51	0.35±0.40
Starch 4	34.8±0.71	16.2±0.36	0	10.7±0.34	0.53±0.46
Starch 5	37.0±7.85	15.7±4.65	0	10.4±3.19	0.46±0.40
Starch 6	35.8	17.4	0	9.77	1.05
Starch 7	34.1±3.35	15.2±0.36	0.79±1.11	8.44±0.07	0.30±0.42
Starch 8	54.4±1.65	19.0±0.33	0	12.7±1.01	0.48±0.68
Starch 9	36.4±0.90	17.7±0.43	0	8.41±0.17	0.97±0.02
Starch 10	37.6±0.82	15.5±0.82	0	9.51±0.50	0
Starch 11	22.5±0.26	10.0±0.77	0	4.74±0.25	0
Glucose	31.0±0.08	12.4±1.09	3.05±0.30	7.15±0.02	0.41±0.57

Table 14. Efficiency of starch degradation by the microbiota that colonises animals fed either Waxy starch, Starch nos. 4, 6 or 9.

	Degradation of dietary starch			
Animals fed Starch:	Starch 4	Starch 6	Starch 9	Amylose (Sigma)
1	+	+	+++	+
1	+	-	+	-
4	+++	++	++	+
4	++++	+++	++	++
6	++++	++++	++++	++
6	++++	+	+++	+++
9	+++	+	++++	+++

Example 10

Specific pathogen free (SPF) mice were fed synthetic diets consistent with those presented in Table 6 but using waxy starch and starches 4, 6 and 9 (Table 4). Five animals per group were used and maintained in the diet for 2 weeks. Animals were sacrificed and the gastrointestinal tract was collected. Contents from the stomach, ileum, caecum and colon were collected, weighed and stored on ice for processing within an hour. The major bacterial groups were enumerated using routine selective media. The groups include the obligate anaerobes, lactobacilli, enterococci, coliforms, amylolytic bacteria, clostridia and bifidobacteria. Amylolytic activity was assessed for isolates from the mice on the various diets by ranking the zone of clearance around colonies on agar plates prepared using either amylose (Sigma) of starches 4, 6 or 9. Results are presented in Figures 4, 5, 6 and 7. It can be seen in these figures that the different starches will induce altered levels of specific groups of microbes at different sites in the tract. For example starch 6 and 4 stimulate lactobacillus from the stomach, ileum and caecum; starch

9 stimulates bifidobacterium in all sites samples; starch 4 stimulates endospore forming populations such as the clostridia in all sites sampled and suppresses the bifidobacterial numbers as all sites sampled; starch 9 suppressed endospore forming populations in all regions sampled.

Example 11

Ex-germ free mice colonised with human faecal homogenates were fed a commercial animal diet. Material from gastrointestinal of germ free mice colonised with human microbes (gastric, ileal, caecal and colonic content) was diluted in Wilkins Charlgren broth (1/1000). The faecal microbial composition of the animal that served as a source for inoculum is presented in Table 15. Diluted material was used as the inoculum for the starch media (Table 1) continuing the different resistant starches in Table 4. The mice gastrointestinal content mixes were sampled at 0 and 9 h post inoculation.

The use of different modifications of resistant starches or unmodified starches could be used to control specific populations at different sites. This has been shown when gut contents from the stomach, ileum, caecum or colon of ex-germ-free mice colonised with human colon microflora were collected and inoculated into media containing the various starches as in Table 4. The mixtures were incubated anaerobically at 37°C. The concentrations of the major bacteria groups were enumerated and these included the total anaerobes, lactobacilli, bifidobacteria. It was shown that the modification influenced the levels of the different microbes. For example, starch 9 induced higher levels of obligate anaerobes in the ileum than were induced by starch 8 (Figure 8) while starch 8 promoted higher levels of these obligate anaerobes in the caecum than were induced by starch 9 (Figure 9).

Table 15. Microbial composition of faeces from mouse to be used

Bacteria	CFU per g
Lactobacilli	$<10^3$
Bifidobacteria	$1.7x10^5$
Enterococci	$3.7x10^7$
E. coli	$<10^3$
Total anaerobes	$9.6x10^9$
Total amylolytic endospores	$<10^3$ $3.3x10^3$

The resident bifidobacterial and amylolytic population may be replaced with new bfidobacterial and amylolytic populations. This will happen if the unmodfified Hi-maize™ (Starch no. 6) is supplied. Although bifidobacterial and amylolytic populations will be disadvantaged in the short term, animals fed Starch 6 (fro about 2.5 months) have a dense bifidobacterial and amylolytic population through out the gastrointestinal tract (Figures 4, 5, 6 and 7 and Table 14).

Uses

It has been shown that carboxymethylated resistant starch consumption resulted in greater numbers of faecal *Bacteroides* than unmodified resistant starch. It is well established that *Bacteroides spp* contribute to saccharide degradation in the large intestine, in particular polysaccharides degradation (Salyers, 1979). This would result in an increase in short chain fatty acids, which are used as metabolic fuel for the epithelial mucosa and for the host. In addition, there is a clear link between the levels of butyrate and the iincidence of polyps and cancer (Young, 1996). Consequently, enhancing bacteroides numbers will lead to increased fermentation which will contribute to intestinal health and protect from the risks of colon cancer.

Other chemically modified starches may lead to enhancement of other beneficial bacteria in the large intestine. Consequently, one can use a

modified resistant starch in the diet to achieve one or all of the following conditions:

i) as a general gut microflora stabiliser:

ii) in clinical conditions related to disturbances e.g. flora related irritable bowel syndrome and inflammatory bowel disease, Crohn's disease, diarrhoea;

iii) improved intestinal health e.g. of the epithelial mucosa;

iv) immunostimulating activities; and

v) colon cancer

In addition, as discussed by Coates (Coates, 1988), resistant starch ingestion can cause a lowering of the pH which will lead to suppression of bacterial transformation of cholesterol and bile acids, thus affecting excretion of cholesterol and bile acids. Since the present inventors have found that modification of the resistant starch affected utilisation by specific microbes and the bacterial groups that were enhanced, modifications of the resistant starch could influence cholesterol and bile acid excretion levels.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. An improved method of enhancing a population of one or more target microorganisms in the gastrointestinal tract of an animal, the improvement comprising providing to the animal a selected modified or unmodified resistant starch or mixtures thereof, such that the one or more microorganisms will selectively utilise the starch and/or increase in number and/or activity in the gastrointestinal tract.

2. The method according to claim 1 wherein the resistant starch is selected from high amylose starches and modified forms thereof.

3. The method according to claim 2 wherein the high amylose starch includes maize starch having an amylose content of 50% w/w or more.

4. The method according to claim 3 wherein the maize starch having an amylose content of 80% w/w or more.

5. The method according to claim 2 wherein the high amylose starch includes rice or wheat starch having an amylose content of 27% w/w or more.

6. The method according to claim 2 wherein the high amylose starch includes particular granular size ranges of starches having an amylose content of 50% or more with enhanced resistant starch content.

7. The method according to claim 2 wherein the high amylose starch from plants selected from the group consisting of maize, barley, wheat, rice, legumes, bananas, potatoes, and modified forms thereof.

8. The method according to any one of claims 2 to 7 wherein the resistant starch is modified chemically, enzymatically, and/or physically.

9. The method according to claim 8 wherein the chemical modification is by etherification, esterification, or acidification.

10. The method according to claim 8 wherein the physical modification is by crystallisation.

11. The method according to any one of claims 2 to 7 wherein the modified resistant starch is selected from the group consisting of hydroxypropylated starch, acetylated starch, octenyl succinated starch, carboxymethylated starch, and succinated starch.

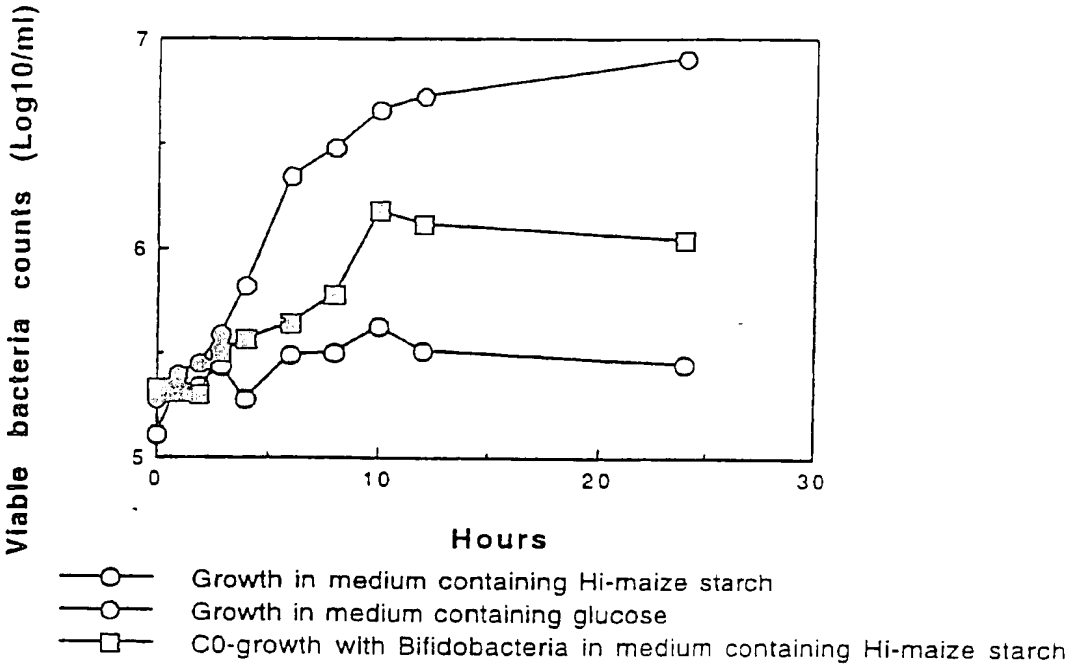

Figure 1

Lactobacillus Pond

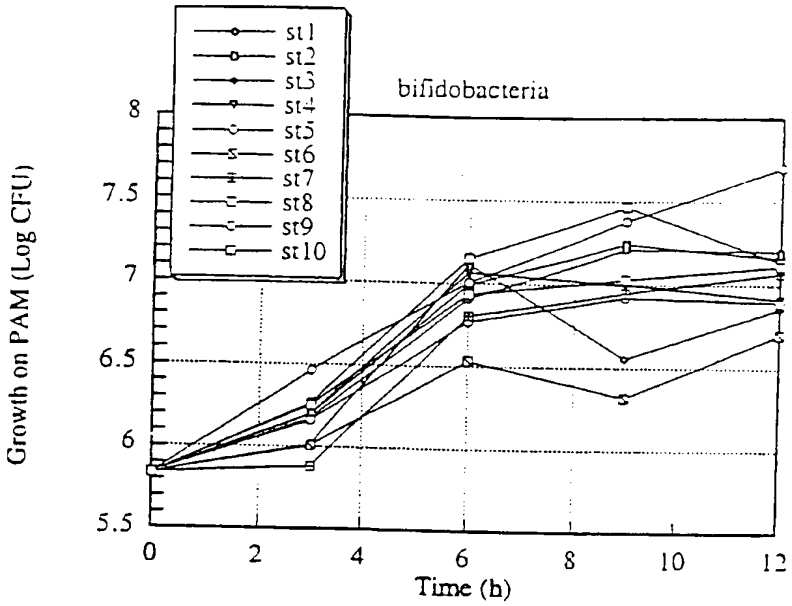

Figure 2

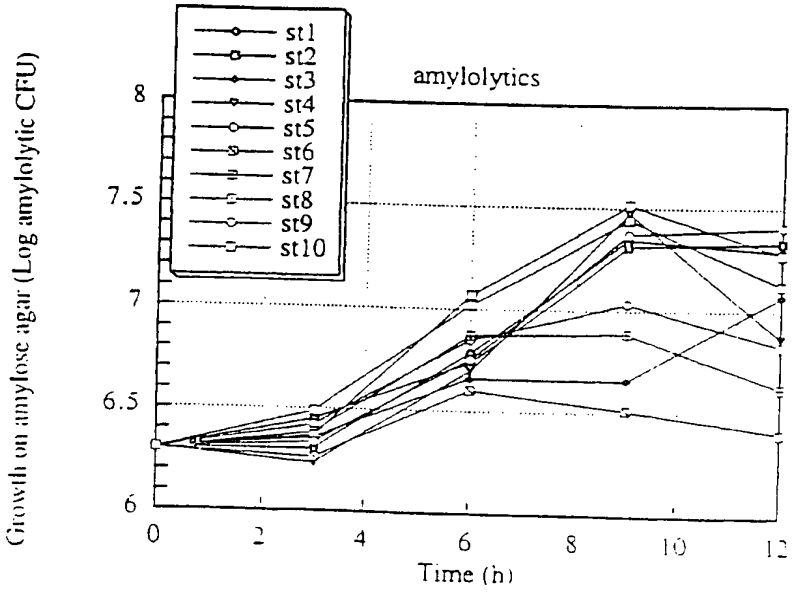

Figure 3

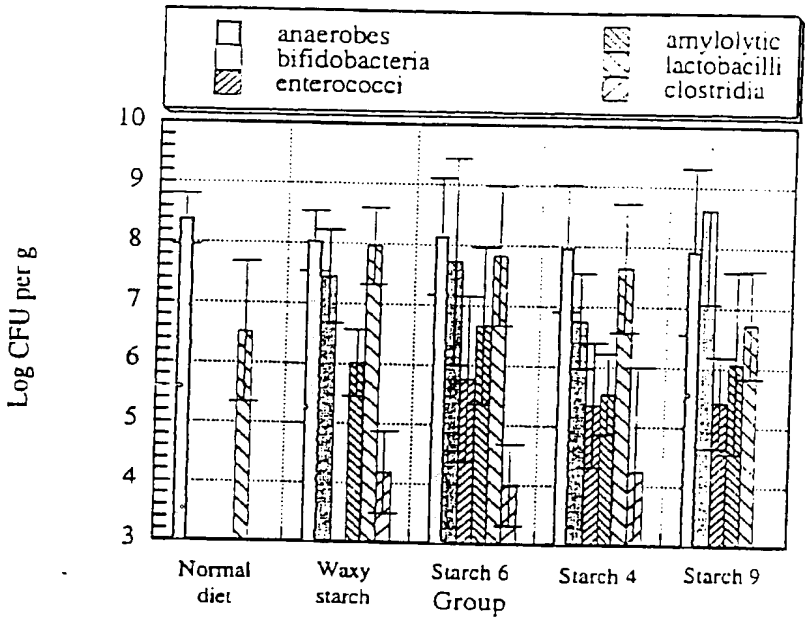

Figure 4

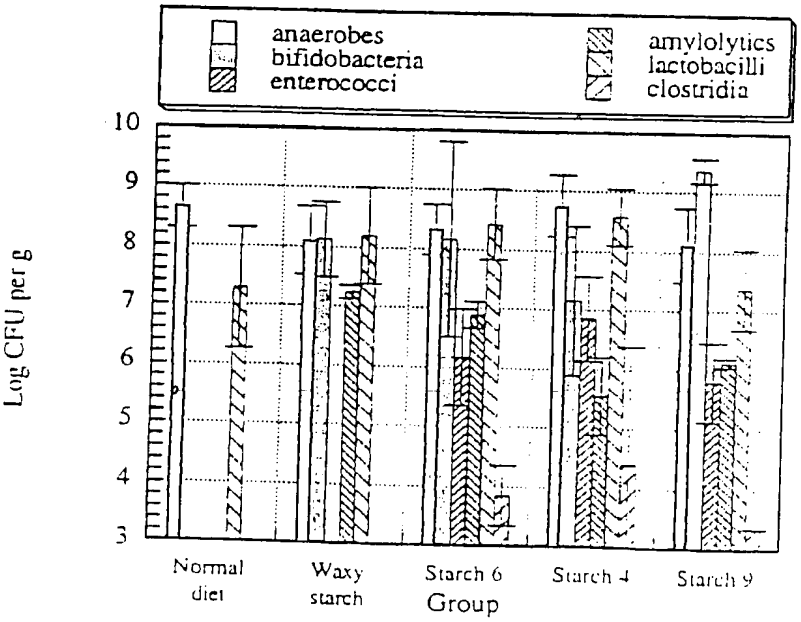

Figure 5

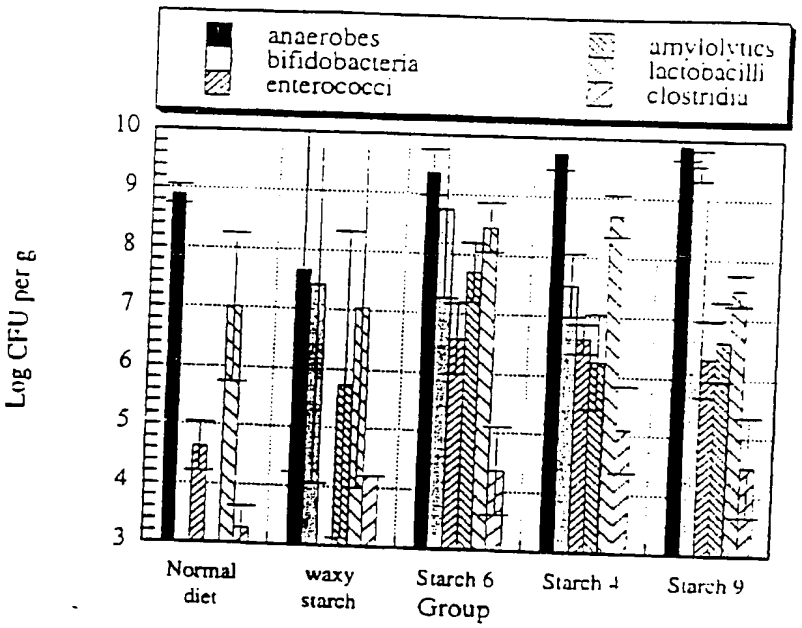

Figure 6

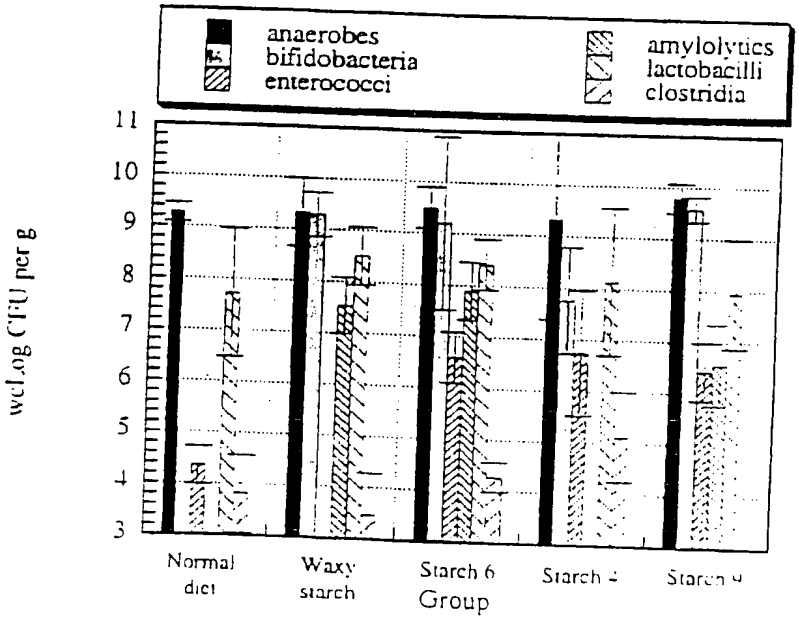

Figure 7

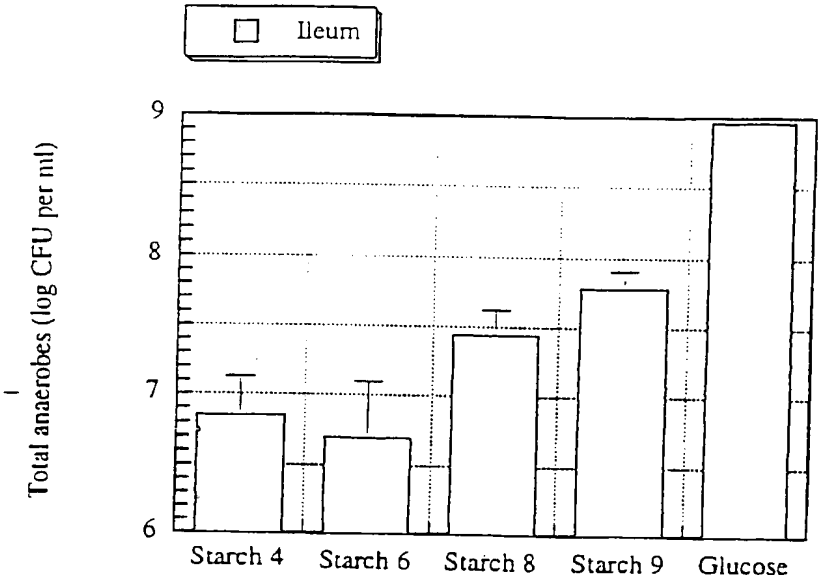

Figure 8

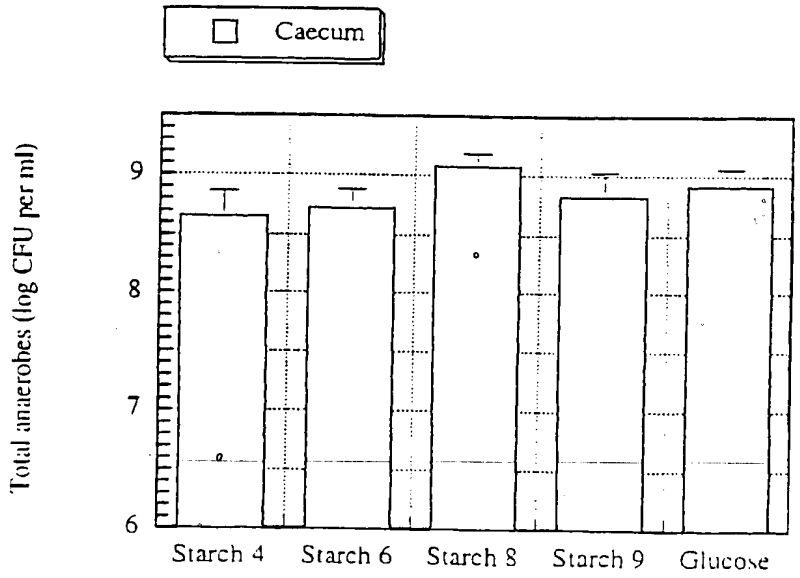

Figure 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/AU 97/00175

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6] A61K 31/175 35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61K 31/175 35/78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent and Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO, A, 96/08261 (The University of New South Wales et al) 21 March 1996	1-11
X	EP, A, 0659769 (MATSUTANI CHEMICAL INDUSTRY CO. LTD) 28 June 1995	1-9
X	AU, B, 21247/67 (The Green Cross Corporation) 7 November 1968	1-2, 6-8

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1997	27 JUN 1997
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (06) 285 3929	Authorized officer H. Flame-Hiotakis Telephone No. (06) 283 2103

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/AU 97/00175

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5147668 (Munk) 15 September 1992	1-7
X	Nutrition Reports International, Volume 15, Number 2, February 1977, Bruns et al., "Effect of Modified Starch on the Microflora of the Small Intestine and Caecum of Rats", pages 131-138	1-2, 6, 8, 11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/AU 97/00175

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information

Patent Document Cited in Search Report		Patent Family Member					
WO, A	96/08261	AU, A	35579/95	CA, A	2199140		
EP, A	0659769	JP, A	7170938				
US, A	5147668	DE, A	3727946	DE, A	3879808	DE	8810216
		EP, A	303946	JP, A	1095726		

END OF ANNEX